

***Cross-reference to related application***

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GRAS status. Thus they are less desirable than the exopolysaccharides of microorganisms, such as lactic acid bacteria, which have the GRAS status.

The exopolysaccharides produced by lactic acid bacteria can be divided in two groups, heteropolysaccharides and homopolysaccharides; these are synthesized by totally different mechanisms. The former consist of repeating units in which residues of different types of sugars are present and the latter consist of one type of monosaccharide. The synthesis of heteropolysaccharides by lactic acid bacteria, including lactobacilli, has been studied extensively in recent years. Considerable less information is present on the synthesis of homopolysaccharides from lactobacilli, although some studies have been performed. The information on the synthesis of homopolysaccharides in lactobacilli is mainly limited to the synthesis of glucans and only two reports, written by the present inventors, exist on the synthesis of fructans. In one of these reports the *Lactobacillus reuteri* strain LB 121 was found to produce both a glucan and a fructan when grown on sucrose, but only a fructan when grown on raffinose (van Geel-Schutten, G.H. *et al.*, Appl. Microbiol. Biotechnol. (1998) 50, 697-703). In the other report was found that *Lactobacillus reuteri* strain LB 35-5, a spontaneous mutant of *Lactobacillus reuteri* strain LB 121, only produced a glucan when grown on sucrose (van Geel-Schutten, G.H. *et al.*, Appl. Environ. Microbiol. (1999) 65, 3008-3014). In the other report the soluble glucan and fructan were also characterised by their molecular weights (of 3,500 and 150 kDa respectively) and the glucan was reported to be highly branched with a unique structure consisting of a terminal, 4-substituted, 6-substituted, and 4,6-di-substituted  $\alpha$ -glucose in a molar ratio 1.1 : 2.7 : 1.5 : 1.0 (van Geel-Schutten, G.H. *et al.*, Appl. Environ. Microbiol. (1999) 65, 3008-3014). No structural identification of a similar glucan produced by a *Lactobacillus* had been reported before. The fructan was identified as a (2 $\rightarrow$ 6)- $\beta$ -D-fructofuranan (also called a levan). This was the first example of levan synthesis by a *Lactobacillus* species.

### ***Summary of the invention***

A novel enzyme having glucosyltransferase activity using sucrose as a substrate has now been found in *Lactobacillus reuteri*, and its amino acid sequence and other structural properties have been determined. The enzyme is unique in that it is capable of producing a highly branched glucan with  $\alpha$ -1,4- and  $\alpha$ -1,6- glucosidic links. The invention thus pertains to an enzyme, to DNA encoding it, to cells containing such DNA

and to their use in producing carbohydrates, as defined in the appending claims. The invention also pertains to glucans so produced as well as to oligosaccharides and chemically derivatised glucans.

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### Description of the invention

It was found according to the invention that the glucans are produced by certain *Lactobacillus* strains, in particular by certain strains of *Lactobacillus reuteri*, as a result of the activity of a single glucosyltransferase (glucansucrase).

10 The nucleotide and amino acid sequence of the novel glucosyltransferase are represented by SEQ ID No. 1 and 2, respectively, and are also shown in figure 3.

15 The start codon of the glucosyltransferase is preceded by a putative ribosome binding site with the nucleotide sequence GAAGGAGA (located 5 base pairs upstream of the start codon of the glucosyltransferase; see SEQ ID No. 1 and figure 3). Furthermore, the start codon is preceded by the nucleotide sequence TATAAT, also called Pribnow box or -10 region, (located 42 base pairs upstream of the start codon; see SEQ ID No. 1 and figure 3) and by the nucleotide sequence TTGAAA, also called -35 region (located 80 base pairs upstream of the start codon; see SEQ ID No. 1 and figure 3).

20 The invention covers a protein having glucosyltransferase activity with sucrose as substrate comprising an amino acid sequence with an amino acid identity of at least 50%, preferably at least 60%, and more preferably at least 70%, compared to the amino acid sequence 531-1781 of SEQ ID No. 2. The invention also covers a part of a protein with at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence 531-1781 of SEQ ID No. 2. The present invention covers a protein having glucosyltransferase activity with sucrose as substrate with an amino acid identity of at least 50%, preferably at least 60%, and more preferably at least 70%, compared to the amino acid sequence of SEQ ID No. 2. The invention also covers a part of a protein with at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence of SEQ ID No. 2. The novel glucosyltransferase has homology with several other proteins as revealed by amino acid sequence alignment. A high homology (figure 5) was found with an alternansucrase of *Leuconostoc mesenteroides* strain NRRL B-1355 (46% identity, within 1261 amino acids of amino acid sequence

531-1781 of SEQ ID No. 2) and a dextransucrase of *Leuconostoc mesenteroides* strain NRRL B-512F (44% identity, within 1270 amino acids of amino acid sequence 531-1781 of SEQ ID No. 2). Furthermore, the alignment revealed the presence of various domains also found in the other glucosyltransferases, such as an N-terminal variable domain, a catalytic domain and a C-terminal glucan binding domain. The N-terminal domain shows almost no identity with the N-terminal domains of other glucosyltransferases and an N-terminal signal peptide could not be detected.

The invention also covers a protein comprising an amino acid sequence of at least 100 amino acids, exhibiting at least 55%, preferably at least 65% amino acid identity with the corresponding part of the amino acid sequence 972-1514 (catalytic domain) of SEQ ID No. 2. The catalytic domain shows a high level of homology (about 50% identity) with other known streptococcal and *Leuconostoc* glucosyltransferases and putative functions based on the alignment can be ascribed to several amino acids within this catalytic domain (figure 4). Asp-1024, Glu-1061 and Asp-1133 of SEQ ID No. 2 are putative catalytic residues, Asp-984 of SEQ ID No. 2 is a putative calcium binding residue and Arg-1022 of SEQ ID No. 2 a putative chloride binding residue. His-1132 and Gln-1514 of SEQ ID No. 2 may stabilize the transition state and the residues Asp-1027, Asn-1028, Asp-1062 and Trp-1063 of SEQ ID No. 2 may play a role in binding of acceptor molecules and in the transfer of the glucosyl moiety.

The invention further covers a protein comprising an amino acid sequence of at least 100 amino acids, exhibiting at least 50%, preferably at least 60%, amino acid identity with the corresponding part of the amino acid sequence 1515-1781 (glucan binding domain) of SEQ ID No. 2. The C-terminal putative glucan binding domain is much shorter than the corresponding domains in other glucosyltransferases but three known repeats, resembling YG repeats, are described: YYFYDLAGNMVKN (SEQ ID No. 3) starting at amino acid position 1656 of SEQ ID No. 2, WYFFDQDGKMVEN (SEQ ID No. 4) starting at amino acid position 1678 of SEQ ID No. 2 and TYYFDNYGKMVRN (SEQ ID No. 5) starting at amino acid position 1725 of SEQ ID No. 2. YG repeats are defined by the presence of one or more aromatic residues (of which one is usually tyrosine), followed by 3-4 glycine residues downstream, a hydrophobic residue, a neutral polar residue (usually glycine or asparagine) and 1-3 hydrophobic residues. It is striking that the number of repeats necessary to ensure glucan binding properties is different for enzymes producing a soluble or an insoluble glucan. Possibly the glucan binding domain is also involved in the determination of the glucan

structure and the polymer chain growth. Furthermore, this domain seems also necessary for the complete glucosyltransferase activity.

Specific amino acids of the glucosyltransferase that are believed to be important for the unique properties of the enzyme include Pro-1026, Ile-1029, Met-1034, Asn-1035, Ser-1136, Ala-1143, Ile-1170, Leu-1223, Ala-1413, Val-1418, Ala-1428, Leu-1442 of the amino acid sequence of SEQ ID No. 2. So a protein, mutant or part thereof, comprising at least one of the above mentioned amino acids is also part of the invention. Particularly Pro-1026 and Ile-1029 are of interest. Pro-1026 is found in a position where a conserved Val is found in other glucosyltransferases. Compared with Val, the presence of Pro results in a more rigid protein structure. This change of protein structure might influence the glucosidic bonds formed and might explain the unique structure of the glucan. Ile-1029 is also found in a position where a conserved Val is present in other LAB glucosyltransferases not producing  $\alpha(1,4)$  bonds. An identical amino acid substitution is observed in amylosucrase, a glucosyltransferase synthesizing  $\alpha(1,4)$  bonds.

~~A nucleotide sequence encoding any of the above mentioned proteins, mutants, variants or parts thereof is also a subject of the invention. Furthermore, the nucleic acid sequences corresponding to expression-regulating regions (promoters, enhancers, terminators) contained in the nucleic acid sequence (1)-(160) or (5507)-(6026) of SEQ ID No. 1 (see also figure 3) can be used for homologous or heterologous expression of genes. Such expression-regulating sequences are operationally linked to a polypeptide-encoding nucleic acid sequence such as the genes of the glucosyltransferase according to the invention. Inverted repeats are located 62 base pairs downstream the termination codon (TAA), suggesting the presence of a Rho independent transcription termination signal. The -10 and -35 consensus promoter sequences, two motifs generally present upstream of the start codon of procaryotes, are identified as described above. Other promoter, enhancer or terminator were not identified. A nucleic acid construct comprising the nucleotide sequence operationally linked to an expression-regulating nucleic acid sequence is also covered by the invention.~~

A recombinant host cell, such as a mammalian (with the exception of human), plant, animal, fungal or bacterial cell, containing one or more copies of the nucleic acid construct mentioned above is an additional subject of the invention. The glucosyltransferase gene has been cloned and expressed in *E. coli*. The molecular weight predicted from the first deduced amino acid sequence (*i.e.* the amino acid sequence 531-

1781 of SEQ ID No. 2; see examples) of the recombinant glucansucrase expressed in *E. coli* is 145 kDa.

The invention further covers a enzymatically active protein as defined above which, in the presence of sucrose, produces a glucan having 38-48% 4-linked anhydroglucose units, 17-28% 6-linked anhydroglucose units, and 7-20% 4,6-linked anhydroglucose units, preferably a glucan having 40-46% 4-linked anhydroglucose units, 19-26% 6-linked anhydroglucose units, and 9-18% 4,6-linked anhydroglucose units. There is a large variation in glucans due to differences in the type of bonds present, degree and type of branching, length of the glucan chains, molecular weight, and the conformation of the polymers. The structure of this glucan is unique in that it is highly branched, consists of terminal, 4-substituted, 6-substituted, and 4,6-di-substituted  $\alpha$ -glucose in a molar ratio 1.1 : 2.7 : 1.5 : 1.0 and has a high molecular weight of 3500 kDa. The novel glucan may be synthesized by a glucosyltransferase present in the *Lactobacillus* strains, preferably *Lactobacillus reuteri* strains and more preferably *Lactobacillus reuteri* strains LB 121 and LB 35-5. *Lactobacillus reuteri* belongs to the group of lactic acid bacteria which are known to play an important role in the fermentative production of food and feed. Because of this, lactic acid bacteria are food-grade micro-organisms that possess the Generally Recognised As Safe (GRAS) status.

The invention also pertains to a process of producing a glucan as described above. This glucan can be produced by a *Lactobacillus* strain, preferably a *Lactobacillus reuteri* strain, and more preferably *Lactobacillus* strain LB 121 or LB 35-5 or by a recombinant micro-organism expressing the glucosyltransferase according to the invention or by an isolated glucosyltransferase according to the invention and a suitable glucose source such as for instance sucrose. The glucosyltransferase may be isolated by conventional means from the culture of a glucosyltransferase-positive lactic acid bacterium, especially a *Lactobacillus reuteri*, or from a recombinant organism expressing the glucosyltransferase gene.

Additionally, the invention concerns a process of producing gluco-oligosaccharides containing the characteristic structure of the glucan described above using an isolated glucosyltransferase according to the invention or a *Lactobacillus* strain, preferably a *Lactobacillus reuteri* strain, or a recombinant micro-organism containing a glucosyltransferase according to the invention. There is a growing interest in oligosaccharides derived from homopolysaccharides, for instance for prebiotic purposes. Several fructo- and gluco-oligosaccharides are known to stimulate the growth of

bifidobacteria in the human colon. Gluco-oligosaccharides produced by the glucosyltransferase described above can be used as prebiotics and probiotics and are also part of the invention. The production of the gluco-oligosaccharides is different from the glucan synthesis reaction. In addition to sucrose, the substrate of the glucosyltransferase, an acceptor molecule such as maltose or lactose is necessary for the acceptor reaction. Another way of producing gluco-oligosaccharides is by hydrolysis of the glucan described above. This hydrolysis can be performed by known hydrolysis methods such as enzymatic hydrolysis with enzymes such as amylase, dextranase or pullulanase or by acid hydrolysis. The produced gluco-oligosaccharides must contain at least one 1,6- or one 4,6- glucosidic link to be used as prebiotics.

The invention also covers a glucan having 38-48% 4-linked anhydroglucose units, 17-28% 6-linked anhydro-glucose units, and 7-20% 4,6-linked (branching) anhydro-glucose units, preferably a glucan having 40-46% 4-linked anhydroglucose units, 19-26% 6-linked anhydroglucose units, and 9-18% 4,6-linked anhydroglucose units and a gluco-oligosaccharide containing at least two 4-linked anhydroglucose units, at least one 6-linked anhydroglucose units and at least one 4,6-double linked anhydroglucose units. The novel gluco-oligosaccharides contain at least 5, preferably at least 6 or even at least 8 anhydroglucose units. In addition, they may contain one non-glucose terminal unit such as galactose, mannose or fructose. The glucan and the gluco-oligosaccharides described above can be recovered from the culture supernatant of *Lactobacillus* strains, preferably *Lactobacillus reuteri* strains, and more preferably *Lactobacillus reuteri* strains LB 121 and LB 35-5, containing the glucosyltransferase according to the invention. The glucan can comprise at least 20, up to about 100,000  $\alpha$ -anhydroglucose units with the unique structure described above. The molecular mass of the glucan synthesized by the *Lactobacillus* strains LB 121 and LB 35-5 was 3,500 kDa.

The invention also concerns chemically modified glucans and gluco-oligosaccharides based on the 1,4/1,6- $\alpha$ -glucans described above. Chemical modification can be achieved by oxidation, such as hypochlorite oxidation resulting in ring-opened 2,3-dicarboxy-anhydroglucose units (see e.g. EP-A-427349), periodate oxidation resulting in ring-opened 2,3-dialdehyde-anhydroglucose units (see e.g. WO 95/12619), which can be further oxidised to (partly) carboxylated units (see e.g. WO 00/26257), TEMPO-mediated oxidation resulting in 6-carboxy-anhydroglucose units (see e.g. WO 95/07303). The oxidised glucans have improved water-solubility, altered viscosity and a retarded fermentability and can be used as metal-complexing agents, detergent additives,

strengthening additives, bioactive carbohydrates, emulsifiers and water binding agents. They can also be used as starting materials for further derivatisation such as cross-linking and the introduction of hydrophobes. Oxidised glucans coupled to proteins can be used as emulsifiers and stabilizers. (Partial) hydrolysis of glucans according to the invention  
5 and/or modified glucans results in gluco-oligosaccharides, which can be used as bioactive carbohydrates or prebiotics. The oxidised glucans of the invention preferably contain 0.05-1.0 carboxyl groups per anhydroglucose unit, *e.g.* as 6-carboxyl units.

Another type of chemical modification is phosphorylation, as described in O.B. Wurzburg (1986) Modified Starches: properties and uses. CRC Press Inc., Boca Raton,  
10 97-112. One way to achieve this modification is by dry heating glucans with a mixture of monosodium and disodium hydrogen phosphate or with tripolyphosphate. The phosphorylated glucans are suitable as wet-end additives in papermaking, as binders in paper coating compositions, as warp sizing-agents, and as core binders for sand molds for metal casting. A further type of derivatisation of the glucans is acylation, especially  
15 acetylation using acetic or propionic anhydride, resulting in products suitable as bleaching assistants and for the use in foils. Acylation with *e.g.* alkenyl succinic anhydrides or (activated) fatty acids results in surface-active products suitable as *e.g.* surfactants, emulsifiers, and stabilizers.

Hydroxyalkylation, carboxymethylation, and amino-alkylation are other methods  
20 of chemical derivatisation of the glucans. Hydroxyalkylation is commonly performed by base-catalysed reaction with alkylene oxides, such as ethylene oxide, propylene oxide or epichlorohydrine; the hydroxyalkylated products have improved solubility and viscosity characteristics. Carboxymethylation is achieved by reaction of the glucans with monochloroacetic acid or its alkali metal salts and results in anionic polymers suitable for  
25 various purposes including crystallisation inhibitors, and metal complexants. Amino-alkylation can be achieved by reaction of the glucans with alkylene-imines, halo-alkyl amines or amino-alkylene oxides, or by reaction of epichlorohydrine adducts of the glucans with suitable amines. These products can be used as cationic polymers in a variety of applications, especially as a wet-end additive in paper making to increase  
30 strength, for filler and fines retention, and to improve the drainage rate of paper pulp. Other potential applications include textile sizing and wastewater purification. The above mentioned modifications can be used either separately or in combination depending on the desired product. Furthermore, the degree of chemical modification is variable and depends on the intended use. If necessary 100% modification, *i.e.* modification of all



anhydroglucose units can be performed. However, partial modification, e.g. from 1 modified anhydroglucose unit per 100 up to higher levels, will often be sufficient in order to obtain the desired effect.

Use of a *Lactobacillus* strain capable of producing the novel and unique glucan is also covered by the invention. Preferably, the strain is also capable of producing a fructan, which can be either a levan, inulin or both. More preferably, the strain is also capable of producing fructo-oligosaccharides. For producing a glucan and/or fructan mentioned above a *Lactobacillus* strain according to the present invention uses preferably sucrose as a substrate. The glucan according to the invention can be produced when the *Lactobacillus* strain is cultured in the presence of sucrose and/or after the *Lactobacillus* strain was cultured in the presence of sucrose during a sufficient time period. The *Lactobacillus* strains according to the invention are deposited at the BCCM/LMG Culture Collection (Gent, Belgium) under the deposit numbers LMG P-18388 (*Lactobacillus reuteri* wild-type strain LB 121) and LMG P-18389 (*Lactobacillus reuteri* mutant strain LB 35-5). The efficacy of some *Lactobacillus reuteri* strains as a probiotic has been demonstrated in various animals such as for instance poultry and humans. The administration of *Lactobacillus reuteri* to pigs resulted in significantly lower serum total and LDL-cholesterol levels, while in children *Lactobacillus reuteri* is used as a therapeutic agent against acute diarrhea. For this and other reasons *Lactobacillus reuteri* has already been supplemented to commercially available probiotic products. The mode of action of *Lactobacillus reuteri* as a probiotic is still unclear. Preliminary studies indicated that gut colonization by *Lactobacillus reuteri* may be of importance. According to the invention, it was found that the mode of action of *Lactobacillus reuteri* as a probiotic may reside partly in the ability to produce polysaccharides. *Lactobacillus* strains, preferably *Lactobacillus reuteri* strains, more preferably *Lactobacillus reuteri* strains LB 121, LB 35-5 and other strains capable of producing a glucan having 38-48% 4-linked anhydroglucose units, 17-28% 6-linked anhydroglucose units, and 7-20% 4,6-linked anhydroglucose units, preferably a glucan having 40-46% 4-linked anhydroglucose units, 19-26% 6-linked anhydroglucose units, and 9-18% 4,6-linked anhydroglucose units can thus advantageously be used as a probiotic. They can also, together with these polysaccharides, be used as a symbiotic (instead of the term symbiotic, the term synbiotic can also be used).

In that respect, a further aspect of the invention relates to a probiotic or symbiotic composition containing a *Lactobacillus* strain capable of producing a glucan and/or

gluco-oligosaccharide according to the invention. Preferably, said strain also produces a fructan. The probiotic or symbiotic compositions of the invention may be directly ingested with or without a suitable vehicle or used as an additive in conjunction with foods. They can be incorporated into a variety of foods and beverages including, but not limited to, yoghurts, ice creams, cheeses, baked products such as bread, biscuits and cakes, dairy and dairy substitute foods, confectionery products, edible oil compositions, spreads, breakfast cereals, juices and the like.

Another aspect of the invention pertains to a process of improving the microbial status in the mammalian colon comprising administering an effective amount of a *Lactobacillus* strain capable of producing a glucan and/or gluco-oligosaccharide according to the invention and/or a fructan.

Furthermore, a process of improving the microbial status of the mammalian colon comprising administering an effective amount of a glucan or gluco-oligosaccharide according to the invention is also a part of the present invention.

### **Examples**

Example 1: Isolation of DNA from *Lactobacillus reuteri*, nucleotide sequence analysis of the glucosyltransferase gene, construction of plasmids for expression of the glucosyltransferase gene in *E. coli* DH5 $\alpha$ , expression of the glucosyltransferase gene in *E. coli* DH5 $\alpha$ , and identification of the novel glucan produced by the recombinant enzyme.

General procedures for cloning, DNA manipulations and agarose gel electrophoresis were essentially as described by Sambrook *et al.* (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York. Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers. DNA was amplified by PCR techniques using ampliTAQ DNA polymerase (Perkin Elmer). DNA fragments were isolated from agarose gels using the Qiagen extraction kit (Qiagen GMBH), following the instructions of the suppliers. *Lactobacillus reuteri* strains were grown anaerobically at 37°C in MRS medium (DIFCO) or in MRS-s medium (MRS medium containing 100 g/l sucrose instead of 20 g/l glucose) and *E. coli* strains were grown aerobically at 37°C in LB medium containing 100  $\mu$ g/ml ampicillin (when appropriate 40  $\mu$ g/ml X-gal was added).

For the isolation of chromosomal DNA, *Lactobacillus reuteri* 121 was grown overnight at 37°C in MRS broth (Difco) supplemented with 40 mM DL-threonine. Cells of 4 ml culture were harvested by centrifugation and resuspended in 10 ml MRS broth supplemented with 40 mM DL-threonine and incubated for 2 h at 37°C. After centrifugation the cells were resuspended in 400 µl protoplast buffer (10 mM sodium maleate, pH 6.1 supplemented with 0.3 M lactose, 10 mM MgCl<sub>2</sub>, 12% polyethyleneglycol 2000, 0.1 M EDTA, 5 mg/ml lysozyme (47,000 U/mg) and 10 U/ml mutanolysine) and incubated for 1 h at 37°C. After centrifugation (1 min, Eppendorf centrifuge), protoplasts were resuspended in 500 µl 20 mM Tris-HCl, pH 8.0. Subsequently, 100 µl laurylsarcosine and 150 µl 5 M NaCl were added and DNA was extracted. Plasmid DNA of *Lactobacillus reuteri* was isolated using a modification of the methods of Anderson and Mc Kay (1983) Appl. Environ. Microbiol. 46, 549-552 and Burger and Dicks (1994) Biotechnol. Technol. 8, 769-772. Fresh prewarmed (37°C) MRS broth (10 ml) was inoculated with 200 µl of an overnight culture and incubated for 2.5 h at 37°C. Cells were harvested by centrifugation and washed with 2 ml sterile STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). After centrifugation, the pellet was resuspended in 380 µl solution I (0.5 M sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8, containing 2 mg/ml lysozyme and 6.6 U mutanolysin). After an incubation of 1.5 h at 37°C, 50 µl of solution II (50 mM Tris-HCl, pH 8.0, 0.25 M EDTA) and 30 µl of solution III (50 mM Tris-HCl, pH 8, 20 mM EDTA, 20% SDS) were added and the suspension was mixed. Sodiumhydroxide (30 µl of a 3 M solution) was added, followed by 50 µl 2 M Tris-HCl and 72 µl 5 N NaCl. After extraction with equal volumes of phenol and chloroform, the DNA was precipitated with ethanol.

The glucosyltransferase (*gtfA*) gene was identified by amplification with PCR using degenerated primers (GTFpr1, 5'GAYAAKWSNAAKSYNRTNGTNSARGC3' (SEQ ID No. 6) and GTFpr2, 5'GNKCNCANATRATRCCNCTRNA3' (SEQ ID No. 7); Y=T or C, K=G or T, W=A or T, S=C or G, R=A or G, N= A, C, G, or T) based on conserved amino acid sequences deduced from different glucosyltransferase genes (*gtfS* of *Streptococcus downei*, *gtfC* of *S. mutans*, *gtfI* of *S. downei*, *gtfK* and *gtfM* of *S. salivarius* and *dsrA* of *Leuconostoc mesenteroides*) and *Lactobacillus reuteri* chromosomal DNA as template. An amplification product with the predicted size of about 660 bp was obtained (figure 1A). To investigate the possible presence of multiple copies of the glucosyltransferase gene, Southern hybridization was performed. DNA was restricted

with endonucleases, separated by agarose gel electrophoresis and transferred to a Hybond nylon membrane. For hybridization, probes were labelled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  using Random Primed DNA labeling kit (Boehringer Mannheim), following the manufacturer's instructions. The Southern hybridization of chromosomal DNA of the *Lactobacillus reuteri* strain 121 with the amplified 660 bp PCR fragment, followed by washing under non-stringent conditions (45°C, 0.5x SSC/0.1 SDS) revealed one hybridizing fragment, suggesting the presence of only a single copy of a glucosyltransferase gene in the *Lactobacillus reuteri* strains. The 660 bp fragment was cloned in *E. coli* JM109 using the pCR2.1 vector. Transformations were performed by electroporation using the BioRad gene pulser apparatus at 2.5 kV, 25  $\mu\text{F}$  and 200  $\Omega$ , following the instructions of the manufacturer. The fragment was sequenced by the method of Sanger *et al.* (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467, confirming that the correct part of the *gtfA* gene had been isolated. The 660 bp amplified fragment was used to design primers for inverse PCR. Using inverse PCR techniques a 3 kb fragment of the isolated *gtfA* gene was generated (figure 1B). This 3 kb amplicon was identified by sequencing and probes were designed to isolate the *EcoRI/BglII* and *EcoRI/HindIII* fragments from a partial DNA library of *Lactobacillus reuteri* in *E. coli* DH5 $\alpha$  (figure 1C). Positive clones were selected by colony blot hybridization using Hybond-N filters, following the instructions of the supplier and the cloned fragments were sequenced. Attempts to clone the C-terminal part of the glucansucrase gene in *E. coli* DH5 $\alpha$  or JM109 using a partial DNA library strategy with different vectors failed. Therefore, the C-terminal part was isolated by inverse PCR. The remaining fragment, located between the *EcoRI/BglII* and *EcoRI/HindIII* fragments, was isolated by PCR techniques (figure 1D). The amplicons obtained were sequenced directly. To eliminate errors due to the PCR reaction, these fragments were sequenced for at least 4 times, using different clones per PCR reaction. Both DNA strands of the entire glucosyltransferase gene were sequenced twice. In this way the sequence of a 5.5 kb region of the *Lactobacillus reuteri* chromosomal DNA, containing the *gtfA* gene and its surroundings, were obtained.

The plasmids for expression of the glucosyltransferase gene in *E. coli* DH5 $\alpha$  were constructed as described hereafter. A 4.8 kb fragment, containing the entire glucosyltransferase gene was generated by PCR, using the primers GTFpr3 (5'ACAACCACCA TGGAATTAGG TCGCACTGAT GTAAC3') (SEQ ID No. 8 ) and GTFpr4 (5'GCCAGCTGGA TCCGTCGACT AGTTTATTTT TGATCAAGCA

TCTTACC3') (SEQ ID No. 9 ). Both primers contained suitable restriction enzyme recognition sites at their 5' ends (*Nco*I in GTFpr3 and *Bam*HI and *Sal*I in GTFpr4). Cloning of this PCR fragment in different vectors failed. Therefore, the strategy depicted in figure 2 was followed. Briefly, the PCR product was restricted with *Xba*I/*Pst*I and *Pst*I/*Bam*HI (figure 1; *Bam*HI site was introduced with GTFpr4). The resulting fragments (1503 bp and 2696 bp, respectively) were cloned separately in pBluescriptIISK<sup>+</sup> yielding pBXP1500 and pBPB2700. Ligation of the 2700 bp *Pst*I/*Sal*I fragment isolated from pBPB2700 in pBXP1500, digested with *Pst*I and *Sal*I, yielded pBGTF (7146 bp) in *E. coli* DH5 $\alpha$ . Plasmid DNA of *E. coli* was isolated using the alkaline lysis method of Birnboim and Doly (1979) Nucleic. Acid Res. 7, 1513-1523 or with a Qiagen plamid kit following the instructions of the supplier. Cells of *E. coli* DH5 $\alpha$  with pBGTF were harvested by centrifugation after 16h of growth. The pellet was washed with 50 mM sodium acetate buffer pH 5.5 containing 1 mM CaCl<sub>2</sub> and 1% (v/v) Tween-80 and the suspension was centrifuged again. Pelleted cells were resuspended in 50 mM sodium acetate buffer pH 5.5 containing 1 mM CaCl<sub>2</sub>, 1% (v/v) Tween-80 and 7.2 mM  $\beta$ -mercaptoethanol. Cells were broken by sonication. Cell debris and intact cells were removed by centrifugation for 15 min at 4°C at 14,000 rpm in an Eppendorf centrifuge and the resulting cell free extract was used in the enzyme assays.

The glucosyltransferase activity was determined at 37°C by monitoring the release of fructose from sucrose or by measuring the amount of glucan produced using *E. coli* cell free extracts or *Lactobacillus reuteri* culture supernatant in reaction buffer (50 mM sodium acetate, 1 mM CaCl<sub>2</sub>, 1% (v/v) Tween-80, 10 g/l sucrose, pH 8). Sucrose, glucose and fructose were determined using commercially available kits. For determination of the molecular weight of the glucosyltransferase produced by *E. coli* or *Lactobacillus reuteri*, SDS-PAGE was performed according to Laemmli (1970) Nature 227, 680-685. SDS-PAGE gels were stained using the PAS activity staining. Glucans were collected by precipitation with ethanol. <sup>1</sup>H-NMR spectroscopy (figure 6) and methylation analysis (table 1) were performed as described by van Geel-Schutten *et al.* (1999) Appl. Environ. Microbiol. 65, 3008-3014. The molecular weights of the glucans were determined by high performance size exclusion chromatography coupled on-line with a multi angle laser light scattering and a differential refractive index detector. After the first nucleotide sequencing of the obtained DNA two putative start codons leading to either a protein encoded by 3834 nucleotides (starting at nucleotide position 1670 of SEQ

ID No. 1) or a protein encoded by 3753 nucleotides (starting at nucleotide position 1751 of SEQ ID No. 1) were identified. Both putative start codons were preceded by a putative ribosome binding site, GCAGG (located 4 base pairs upstream of nucleotide position 1670 of SEQ ID No. 1) or AGAAG (located 14 base pairs upstream of nucleotide position 1751 of SEQ ID No. 1), respectively. At the beginning it was believed that the above mentioned nucleotide forms encoded two glucosyltransferases. Depending on the potential start codon used, one of these glucosyltransferases comprised 1278 amino acids (starting at amino acid positions 504 of SEQ ID No. 2) (3834 nucleotides) and the other comprised 1251 amino acids (starting at amino acid position 531 of SEQ ID No. 2) (3753 nucleotides). The molecular weight (MW) deduced of the amino acid sequence of these glucosyltransferases was 143 and 140 kDa, respectively. The isoelectric point deduced of the amino acid sequence of these glucosyltransferases was 4.73 (for the higher MW protein) and 4.71 (for the lower MW protein), at pH 7, respectively. Surprisingly, the molecular weight of the purified protein from *Lactobacillus reuteri* indicated by SDS-PAGE was not approximately 140 kDa but 180 kDa. After repeating the nucleotide sequencing, it appeared that the above mentioned nucleotide forms did not represent the complete nucleotide sequence of the glucosyltransferase according to the invention, but were merely a part of the complete nucleotide sequence encoding the protein of the invention. The complete nucleotide sequence of the novel glucosyltransferase is represented in SEQ ID No. 1 and the amino acid sequence of said glucosyltransferase is shown in SEQ ID No. 2. All experiments were performed with both the complete nucleotide or amino acid sequence of the protein (SEQ ID No. 1 and 2, respectively) and the partial nucleotide or amino acid sequence mentioned above. The results of the experiments performed with the complete or partial amino acid sequence and the complete or partial nucleotide sequence mentioned above were identical indicating that the part of the glucosyltransferase represented by said partial nucleotide and amino acid sequences is essential for the functionality of the glucosyltransferase according to the invention.

Table 1: Methylation analysis of the glucans produced by *Lactobacillus reuteri* strains and *E. coli* GTFA.

Type of glucosyl units	<i>Lactobacillus reuteri</i> strain 121	<i>Lactobacillus reuteri</i> strain 35-5	<i>E. coli</i> GTFA
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Glc $p$ -(1→	24%	25%	21%
→4)-Glc $p$ -(1→	42%	43%	44%
→6)-Glc $p$ -(1→	22%	21%	24%
→4,6)-Glc $p$ -(1→	12%	11%	11%

Example 2: Adhesion experiments with *Lactobacillus* strains.

The adhesion of *Lactobacillus reuteri* strains to Caco-2 cell lines was determined as described below. Firstly, a bacterial suspension was prepared as follows. *Lactobacillus reuteri* strains LB 121, 35-5, K24 and DSM20016 and *L. rhamnosus* LGG (a well known probiotic strain with good adhering properties) were cultured in MRS broth supplemented with 5  $\mu$ l/ml of methyl-1,2- $^3$ H-thymidine at 37°C for 18-20 h before the adhesion assays. The cultures were harvested by centrifugation, washed with phosphate buffered saline (PBS) and resuspended in PBS or PBS supplemented with 30 g/l sucrose (see Table 2) to a final density of about  $2 \times 10^9$  cfu/ml. Prior to the adhesion assay, the cell suspensions in PBS with 30 g/l sucrose were incubated for 1 hour at 37°C, whereas the cell suspensions in PBS were kept on ice for 1 hour. After incubation at 37°C, the suspensions in PBS with sucrose were centrifuged and the cells were washed with and resuspended in PBS to a final density of about  $2 \times 10^9$  cfu/ml.

Table 2: Incubation of the different *Lactobacillus* strains prior to the adhesion assays.

<i>Lactobacillus</i> strain	Extra incubation	Polysaccharide produced	Group
<i>reuteri</i> 121	PBS sucrose, 37°C for 1 hr	glucan and fructan	As
<i>reuteri</i> 35-5	PBS sucrose, 37°C for 1 hr	glucan	Bs
<i>reuteri</i> K24	PBS sucrose, 37°C for 1 hr	none	Cs
<i>reuteri</i> 121	PBS on ice	none	D
<i>reuteri</i> DSM20016*	PBS on ice	none	E
<i>rhamnosus</i> GG	PBS on ice	none	F

Type strain of *L. reuteri*

Caco-2 cells were cultured as follows. Subcultures of Caco-2 cells (ATCC, code HTB 37, human colon adenocarcinoma), stored as frozen stock cultures in liquid nitrogen were used for the adhesion tests. The Caco-2 cells were grown in culture medium consisting of Dulbecco's modified Eagle medium (DMEM), supplemented with heat-inactivated foetal calf serum (10% v/v), non-essential amino acids (1% v/v), L-glutamine (2mM) and gentamicin (50 µg/ml). About 2,000,000 cells were seeded in 75cm<sup>2</sup> tissue culture flasks containing culture medium and cultured in a humidified incubator at 37°C in air containing 5% CO<sub>2</sub>. Near confluent Caco-2 cell cultures were harvested by trypsinisation and resuspended in culture medium. The number of cells was established using a Bürker-Türk counting chamber.

For the following experiments a Caco-2 monolayer transport system was used. Caco-2 cells cultured in a two-compartment transport system are commonly used to study the intestinal, epithelial permeability. In this system the Caco-2 cell differentiates into polarized columnar cells after reaching confluency. The Caco-2 system has been shown to simulate the passive and active transcellular transport of electrolytes, sugars, amino acids and lipophilic compounds (Hillgren *et al.* 1995, Dulfer *et al.*, 1996, Duizer *et al.*, 1997). Also, a clear correlation between the *in vivo* absorption and the permeability across the monolayers of Caco-2 cells has been reported (Artursson and Karlsson, 1990). For the present transport studies, Caco-2 cells were seeded on semi-permeable filter inserts (12 wells Transwell plates, Costar) at ca. 100,000 cells per filter (growth area ± 1 cm<sup>2</sup> containing 2.5 ml culture medium). The cells on the insert were cultured for 17 to 24 days at 37°C in a humidified incubator containing 5% CO<sub>2</sub> in air. During this culture period the cells have been subjected to an enterocyte-like differentiation. Gentamycin was eliminated from the culture medium two days prior to the adhesion assays.

The adhesion assay was performed as follows. PBS was used as exposure medium. 25 µl of a bacterial suspension ( $2 \times 10^9$  cfu/ml ) were added to 0.5 ml medium. The apical side of the Caco-2 monolayers was incubated with the bacterial suspensions for 1 hour at 37°C. After incubation, remaining fluid was removed and the cells were washed three times with 1 ml PBS. Subsequently, the Caco-2 monolayers were digested overnight with 1 ml 0.1M NaOH, 1% SDS. The lysate was mixed with 10 ml Hionic Fluor scintillation liquid and the radioactivity was measured by liquid scintillation counting using a LKB/Wallac scintillation counter. As a control, the radioactivity of the bacterial suspensions was measured. For each test group, the percentage of bacteria



attached to the monolayers was calculated. All adhesion tests were performed in quadruple. In Table 3 the results of the bacterial adhesion test to Caco-2 cellines are given. From the results can be concluded that the glucans and the fructans contribute to the adherence of *Lactobacillus reuteri* to Caco-2 cellines. This could indicate that

5 *Lactobacillus reuteri* strains producing EPS possess improved probiotic characteristics or that *Lactobacillus reuteri* and its polysaccharides could function as an excellent symbiotic.

Table 3: The results of the bacterial adhesion test to Caco-2 cellines.

Group (see Table 1)	% of bacteria bound to the monolayer
As	6.5
Bs	5.7
Cs	1.8
D	2.3
E	0.9
F	1.3

10 Example 3: Oxidation of glucans.

For TEMPO-mediated oxidation, a glucan produced as described above (dry weight 1 g, 6.15 mmol) was resuspended in 100 ml water. Next, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO; 1% by weight compared to the polysaccharide (0.01 g, 0.065 mmol)) was added and resuspended in 20 min. Sodium bromide (0.75 g, 7.3 mmol) was added and the suspension was cooled to 0°C. The reaction can also be

15 performed without bromide. A solution of hypochlorite (6 ml, 15% solution, 12.6 mmol) was adjusted to pH 10.0 with 3M HCl and cooled down to 0°C. This solution was added to the suspension of the polysaccharide and TEMPO. The course of the reaction was followed by monitoring the consumption of sodium hydroxide solution, which is

20 equivalent to the formation of uronic acid. After 30 min, 59,5 ml 0.1M NaOH was consumed. This amount corresponds to the formation of 96% uronic acid. Thereafter, the solution was poured out in 96% ethanol (comprising 70% of the volume of the solution) causing the product to precipitate. The white precipitate was centrifuged, resuspended in ethanol/water (70/30 v/v) and centrifuged again. Next, the precipitate was resuspended in

25 96% ethanol and centrifuged. The obtained product was dried at reduced pressure. The

uronic acid content was determined by means of the uronic acid assay according to Blumenkrantz and Abdoe-Hansen (Anal. Biochem., 54 (1973), 484). A calibration curve was generated using polygalacturonic acid (5, 10, 15 and 20 µg). With this calibration curve the uronic acid content in a sample of 20 µg of the product was determined. The obtained result was a content of 95% uronic acid with a yield of 95%.

For partial oxidation (about 6%), a glucan produced as described before (dry weight 7.5 g, 46.4 mmol) was resuspended in 50 ml water. Next, TEMPO (1% by weight compared to the polysaccharide (0.075 g, 0.5 mmol)) was added, resuspended in 20 min and cooled to 0°C. A solution of hypochlorite (2.8 ml, 15% solution, 5.9 mmol) was adjusted to pH 9.0 with 3M HCl and cooled down to 0°C. This solution was added to the suspension of the polysaccharide and TEMPO. Within 5 min the mixture became a solid gel.

### *Description of the figures*

SEQ ID No. 2 : The deduced amino acid sequence of glucosyltransferase A (GTFA) of *Lactobacillus reuteri*.

Figure 1: The strategy used for the isolation of the *gtfA* gene from *Lactobacillus reuteri* 121 chromosomal DNA.

Figure 2: The general principle of the construction of the recombinant plasmid with the *gtfA* gene. A PCR product containing the *gtfA* gene was digested with *XbaI* and *PstI* and with *PstI* and *BamHI*. The *XbaI/PstI* (depicted in white) was ligated into the multiple cloning site of pBluescriptIISK<sup>+</sup> in the same direction relatively to the *lacZ* promotor, resulting in pBXP1500. The *PstI/BamHI* part (depicted in grey) was ligated into the multiple cloning site of pBluescriptIISK<sup>+</sup> in the opposite direction relatively to the *lacZ* promotor, resulting in pBPB2700. pBXP1500 was used as a vector for subcloning the C-terminal part of the *gtfA*. pBPB2700 was digested with *PstI* and *SalI* and ligated into pBXP1500, also digested with *PstI* and *SalI*. The resulting plasmid, pBGTF, contained the entire *gtfA* in the same direction relative to the *lacZ* promotor. The ∃ sign indicates the restriction.

Figure 3: The nucleotide and deduced amino acid sequence of *gtfA* of *Lactobacillus reuteri*. The start codon is shown in bold. The putative ribosomal binding site (RBS) is also shown in bold. The nucleotides in italic and underlined upstream of the start codon indicate the -10 region (Pribnow box) and the -35 region. The inverted repeats

(transcription termination) after the stop codon are underlined. The \* sign indicates the stop codon.

Figure 4: Alignment of catalytic cores of alternansucrase (ASR) of *Leuconostoc mesenteroides* strain NRRL B-1355 dextranase (DSRS) of *Leuconostoc mesenteroides* strain NRRL B-512F, glucosyltransferase-D (GTFD) of *Streptococcus mutans* GS5, glucosyltransferase-A of *Lactobacillus reuteri* and amylosucrase (AS) of *Neisseria polysaccharea*. \* indicates identical or conserved residues in all sequences; ---, gap in the sequence; AA amino acids which are conserved in all other glucosyltransferases but not in GTFA; ↓, putative catalytic residues; •, putative calcium binding sites; ♦, putative residues stabilizing the transition state; ∇, residues possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; ◇, putative chloride binding sites; -Ex-, localization of β-strands; -Hx-, localization of α-helices according to Mac Gregor *et al.* (1996) FEBS Lett. 378, 262-266. The numbering of the amino acids of the glucosyltransferase-A of *Lactobacillus reuteri* corresponds to the positions of these amino acids in the amino acid sequence 531-1781 of amino acid sequence SEQ ID No. 2, when the amino acid sequence 531-1781 is renumbered 1-1251.

Figure 4: Dendrogram of glucansucrases of lactic acid bacteria. The horizontal distances are a measure for the difference at the amino acid sequence level. 10% difference is indicated by the upper bar. Bootstrap values (in percentages) are given at the root of each branch.

Figure 5: 500-MHz <sup>1</sup>H-NMR spectra of the glucan produced by *Lactobacillus reuteri* GTFA (A) and by *E. coli* GTFA (B), recorded in D<sub>2</sub>O at 80°C.